

Multigene phylogeny, taxonomy, and potential biological properties of *Pseudorousoella* and *Neorousoella* species (Rousoellaceae, Dothideomycetes) from Asteraceae weeds in northern Thailand

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Abstract

In our study, dead stems of two Asteraceae species (weeds) were collected from northern Thailand. Both morphology and multigene phylogeny were used to determine the identity of the taxa. Maximum likelihood and Bayesian inference analyses of combined LSU, SSU, ITS, *tef1-a* and *rpb2* data revealed two new species *Pseudorousoella bidenticola*, and *Neorousoella chromolaenae* with one new host record of *N. entadae*. Preliminary investigation into antibacterial properties revealed that our three isolates inhibited the growth of *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*. Additionally, we present updated phylogenetic trees for Rousoellaceae, accompanied by detailed descriptions and illustrations of the three identified species.

Key words: 2 new species, antibacterial properties, Ascomycota, *Bidens pilosa*, *Chromolaena odorata*, new host record

Introduction

Asteraceae species exhibit a widespread distribution from polar to tropical regions (Xu et al. 2017). Many of these species hold economic significance, while others are categorized as weeds (Jansen and Palmer 1987; Katinas et al. 2007; Karlsson et al. 2008). In Thailand, numerous invasive weeds have an extensive spread, with *Bidens pilosa* and *Chromolaena odorata* being prevalent at roadsides, disturbed areas, and agricultural lands (Zungsontiporn et al. 2020). Mapook et al. (2020) studied the fungal diversity in *Chromolaena odorata* and provided a global checklist of fungi associated with this plant. Moreover, the information of fungi associated with *Bidens pilosa* was provided in previous studies (Abdou et al. 2010; Guatimosim et al. 2015; Zhang et al. 2018; Li et al. 2020; Htet et al. 2024). However, more knowledge is still needed about the diversity of fungi in Asteraceae plants to understand the fungi associated with this plant family. Moreover, the diversity of fungi in these two invasive weeds in Thailand is being further explored.

Rousoellaceae was introduced by Liu et al. (2014) based on morphology and LSU, ITS, *tef1-α* and *rpb2* sequence data. Members of Rousoellaceae can be found as saprobes and human pathogens (Ahmed et al. 2014; Liu et al. 2014; Mapook et al. 2020; Hyde et al. 2023; Wu et al. 2023). Currently, there are 12 genera in Rousoellaceae, viz., *Appendispora*, *Cytoplea*, *Elongatopedicellata*, *Immorrhia*, *Neorousoella*, *Pararousoella*, *Pseudoneoconiothyrium*, *Pseudorousoella*, *Rousoella*, *Rousoellopsis*, *Setoarthopyrenia*, and *Xenorousoella* (Wijayawardene et al. 2022; Index Fungorum www.indexfungorum.org).

Neorousoella was introduced by Liu et al. (2014) to accommodate a saprobic roussoella-like taxon with the type species *N. bambusae*. The sexual morphology of *Neorousoella* is defined by immersed ascostromata beneath a clypeus or epidermis, appearing as black, dome-shaped, or flattened ovoid structures on the host surface. The asci are bitunicate and cylindrical, while the ascospores are brown or yellowish-brown, ellipsoidal to fusiform, and 2-celled, surrounded by a mucilaginous sheath (Liu et al. 2014). The asexual morphology of *Neorousoella* is characterized by superficial or immersed pycnidia with annellidic, ampulliform, cylindrical conidiogenous cells, producing hyaline, pale brown, oblong to ellipsoidal conidia, each with two guttules (Liu et al. 2014; Jayasiri et al. 2019). Currently, there are 15 epithets listed in the Index Fungorum (www.indexfungorum.org), viz., *Neorousoella alishanensis*, *N. bambusae*, *N. clematidis*, *N. chiangmaiensis*, *N. entadae*, *N. fulvicomae*, *N. heveae*, *N. lenispora*, *N. leucaenae*, *N. lignicola*, *N. magnoliae*, *N. peltophora*, *N. sedimenticola*, *N. solani*, and *N. thailandica*. Recent studies into the genus have been conducted by De Silva et al. (2022) and Hyde et al. (2023).

Pseudorousoella was introduced by Mapook et al. (2020) based on morphology and LSU, SSU, ITS, *tef1-α* and *rpb2* sequence data. The sexual morph of *Pseudorousoella* species is characterized by globose to subglobose, dark brown to black ascomata with an ostiole, comprised of *textura epidermoidea* to *textura angularis* or *textura intricata* cells, with septate, trabeculate pseudoparaphyses, 8-spored, bitunicate, fissitunicate, cylindrical to clavate asci with a pedicel, and uniseriate, hyaline to pale brown, oval to ellipsoid, 1-septate ascospores bearing a gelatinous sheath (Mapook et al. 2020). Asexual morphs of *Pseudorousoella* species are distinguished by solitary, superficial, uni-loculate, globose to obpyriform, pycnidial conidiomata with an ostiole, comprised of *textura angularis* cells, annellidic, ampulliform to oblong, hyaline and unbranched conidiogenous cells and pale brown to reddish brown, aseptate conidia with guttules (Mapook et al. 2020). Currently, two species are listed in the Index Fungorum (www.indexfungorum.org).

Some genera from Rousoellaceae, like *Rousoella* and *Neorousoella*, are recognized for their bioactive secondary metabolites (Takekawa et al. 2013; Phukhamsakda et al. 2018; Chen et al. 2021; Zhong et al. 2021; Sommart et al. 2022). Moreover, the prescreening for antibacterial activity conducted by Mapook et al. (2020) revealed that *Pseudorousoella elaeicola* (MFLUCC 17-1483) inhibits the growth of *E. coli*, resulting in a 10 mm inhibition zone. These findings showed that the species of Rousoellaceae are potential organisms for the production of bioactive secondary metabolites.

In this study, we introduce one new species of *Pseudorousoella* on *Bidens pilosa* (Asteraceae), and one new species with a new host record of *Neorousoella* on *Chromolaena odorata* (Asteraceae). We also provide an updated phylogenetic tree for Rousoellaceae, based on a combined dataset of LSU, SSU,

ITS, *tef1-a* and *rpb2* sequence data. Further, we explore the potential antibacterial activity of our three isolates and discuss their implications for future discoveries of bioactive compounds.

Materials and methods

Sample collection, morphological study and isolation

Dead stems from the Asteraceae plants, *Bidens pilosa* and *Chromolaena odorata*, were collected from northern Thailand. All specimens were brought to the lab in plastic bags labelled with the collection information. Single spore isolation was performed on malt extract agar (MEA) and incubated at 27 °C for 24 hours (Senanayake et al. 2020). The spores were germinated within 24 h using a Motic SMZ 168 Series microscope (Motic Asia, Hong Kong). Germinated spores were transferred to fresh MEA plates. All the detailed morphological characteristics were observed using a Nikon ECLIPSE 80i compound microscope (Nikon, Japan) fitted to a Canon 550D digital camera (Canon, Japan). Tarosoft Image Framework (v 0.9.7) was used to measure photomicrograph structures. Adobe Photoshop CS6 Extended (v 10.0.) was used to edit and prepare photo plates (Adobe system, USA). Forty-day-old cultures were used for molecular studies. Specimens were deposited at the Mae Fah Luang University Herbarium (Herb. MFLU) while living cultures were maintained at Mae Fah Luang University Culture Collection (MFLUCC). Faces of fungi (FoF) and Index Fungorum (IF) numbers were obtained as instructed by Jayasiri et al. (2015) and Index Fungorum (www.indexfungorum.org). Moreover, the species descriptions were submitted to the GMS Microfungi database (Chaiwan et al. 2021).

DNA extraction, PCR amplification and sequencing

Fifty-day-old fungal mycelium was scraped off and placed into a 1.5 ml micro-centrifuge tube using a sterile lancet. Genomic DNA extraction was done using the E.Z.N.A.® Tissue DNA Kit (Omega Biotek Inc., Georgia). Polymerase chain reaction (PCR) was used for DNA amplifications, following the protocols of Mapook et al. (2016). The details of PCR primers and protocols are shown in Table 1. The quality of PCR products was confirmed on 1% agarose gels. Purification and sequencing of PCR fragments with the primers mentioned above were carried out at a commercial sequencing provider (Solgent Co., Ltd, Thailand). The newly generated nucleotide sequences were deposited in the GenBank, and accession numbers were obtained (Table 2).

Table 1. PCR conditions used in this study.

| Gene | Primers | | PCR Conditions |
|--|----------|-----------|--|
| | Forward | Reverse | |
| Large subunit (LSU) | LR0R | LR5 | 95 °C: 3 min, (94 °C: 30 s, 56 °C: 50 s, 72 °C: 1 min) × 40 cycles 72 °C: 7 min. |
| Small subunit (SSU) | NS1 | NS4 | 95 °C: 3 min, (94 °C: 30 s, 55 °C: 50 s, 72 °C :1 min) × 40 cycles 72 °C: 7 min. |
| Internal transcribed spacer (ITS) | ITS5 | ITS4 | 95 °C: 3 min, (94 °C: 30 s, 55 °C: 50 s, 72 °C :1 min) × 40 cycles 72 °C: 7 min. |
| Elongation factor-1 alpha (<i>tef1- a</i>) | EF1-983F | EF1-2218R | 95 °C: 3 min, (94 °C: 30 s, 55 °C: 50 s, 72 °C: 1 min) × 40 cycles 72 °C: 7 min. |
| RNA polymerase II subunit (<i>rpb2</i>) | fRPB2-5F | fRPB2-7cR | 95 °C: 5 min, (95 °C : 1 min, 52 °C: 2 min, 72 °C: 90 s) × 40 cycles 72 °C: 10 min |

Table 2. List of taxa, specimens and sequences used in phylogenetic analyses.

| Species | Strain numbers | GenBank accession numbers | | | | |
|--|-----------------------|---------------------------|-----------------|-----------------|-----------------|-----------------|
| | | ITS | LSU | SSU | <i>tef1- α</i> | <i>rpb2</i> |
| <i>Neorousoella alishanense</i> | FU31016 | MK503816 | MK503822 | MK503828 | – | MN037756 |
| <i>N. alishanense</i> | FU31018 | MK503818 | MK503824 | MK503830 | MK336182 | MN037757 |
| <i>N. bambusae</i> | MFLUCC 11-0124 T | KJ474827 | KJ474839 | – | KJ474848 | KJ474856 |
| <i>N. chromolaenae</i> sp. nov. | MFLUCC 24-0274 | PQ226190 | PQ226193 | PQ226196 | PQ240621 | PQ240623 |
| <i>N. clematidis</i> | MFLUCC 17-2061 | MT310632 | MT214587 | MT226700 | MT394645 | MT394701 |
| <i>N. entadae</i> | MFLUCC 18-0243 | MK347786 | MK348004 | MK347893 | MK360065 | MK434866 |
| <i>N. entadae</i> | MFLUCC 24-0275 | PQ226191 | PQ226194 | PQ226197 | – | PQ240624 |
| <i>N. fulvicomae</i> | MFLUCC 17-2073 | MT310633 | MT214588 | MT226701 | MT394646 | MT394702 |
| <i>N. heveae</i> | MFLUCC 17-1983 | MH590693 | MH590689 | MH590691 | – | – |
| <i>N. lenispora</i> | GZCC 16-0020 T | – | KX791431 | – | – | – |
| <i>N. leucaenae</i> | MFLUCC 18-1544 | MK347767 | MK347984 | MK347874 | MK360067 | MK434876 |
| <i>N. leucaenae</i> | MFLUCC 17-0927 | MK347733 | MK347950 | MK347841 | MK360066 | MK434896 |
| <i>N. lignicola</i> | MUT 5008 | MN556318 | MN556320 | MN556308 | MN605895 | MN605915 |
| <i>N. lignicola</i> | MUT 5373 | KU314953 | MN556321 | KU314954 | MN605896 | MN605916 |
| <i>N. lignicola</i> | MUT 4904 | KT699129 | MN556319 | MN556307 | MN605894 | MN605914 |
| <i>N. magnoliae</i> | MFLU 18-1022 | MK801232 | MK801230 | MK801231 | MK834373 | – |
| <i>N. peltophora</i> | MFLUCC 21-0071 | MZ567051 | MZ567206 | MZ567207 | MZ605441 | MZ605442 |
| <i>N. sedimenticola</i> | CGMCC 3.22470 | OQ798949 | OQ758144 | | OQ809046 | OQ809008 |
| <i>N. sedimenticola</i> | CGMCC 3.22468 T | OQ798948 | OQ758143 | | OQ809045 | OQ809007 |
| <i>N. solani</i> | KT3264 T | LC195218 | LC195209 | LC195206 | LC195212 | – |
| <i>N. solani</i> | KT3265 T | LC195219 | LC195210 | LC195207 | LC195213 | LC195216 |
| <i>N. thailandica</i> | MFLUCC 18-0721 | OL703581 | OL457704 | OL764415 | OM505028 | ON502386 |
| <i>Occultibambusa bambusae</i> | MFLUCC 11-0394 | KU940124 | KU863113 | – | KU940194 | KU940171 |
| <i>O. bambusae</i> | MFLUCC 13-0855 | KU940123 | KU863112 | KU872116 | KU940193 | KU940170 |
| <i>Pseudoneoconiothyrium rosae</i> | MFLUCC 15-0052 T | MG828922 | MG829032 | MG829138 | – | – |
| <i>P. euonymi</i> | CBS 143426 T | MH107915 | MH107961 | – | – | MH108007 |
| <i>P. euonymi</i> | GLMC 1544 | MT153733 | MT156304 | – | – | – |
| <i>Pseudorousoella bidenticola</i> sp. nov. | MFLUCC 24-0273 | PQ226192 | PQ226195 | PQ226198 | PQ240622 | PQ240625 |
| <i>Ps. chromolaenae</i> | MFLUCC 17-1492 T | MT214345 | MT214439 | MT214393 | MT235769 | – |
| <i>Ps. elaeicola</i> | MFLUCC 15-0276a T | MT153733 | MT156304 | – | – | – |
| <i>Ps. elaeicola</i> | MFLUCC 15-0276b | MH742330 | MH742327 | – | – | – |
| <i>Ps. elaeicola</i> | MFLUCC 17-1483 | MT214348 | MT214442 | – | MT235772 | MT235808 |
| <i>Pararousoella mangrovei</i> | MFLUCC 17-1542 | MH025951 | MH023318 | – | MH028246 | – |
| <i>P. mukdahanensi</i> | HKAS 101766 | MH453489 | MH453485 | – | MH453478 | MH453482 |
| <i>P. rosarum</i> | MFLUCC 17-0796 T | MG828939 | MG829048 | NG_061294 | MG829224 | MH028250 |
| <i>Rousoella arundinacea</i> | CPC 35554 | MT223838 | MT223928 | – | MT223723 | – |
| <i>R. bambusarum</i> | GMB0316(HT) | ON479891 | ON479892 | – | ON505015 | ON505011 |
| <i>R. bambusarum</i> | GMB0390 | ON505055 | ON505051 | – | ON505017 | ON505012 |
| <i>R. chiangraina</i> | MFLUCC 10-0556 T | KJ474828 | KJ474840 | – | KJ474849 | KJ474857 |
| <i>R. doimaesalongensis</i> | MFLUCC 14-0584 T | KY026584 | KY000659 | – | KY651249 | KY678394 |
| <i>R. hysterioides</i> | CBS 546.94 T | KF443405 | KF443381 | AB524480 | KF443399 | KF443392 |
| <i>R. intermedia</i> | CBS 170.96 | KF443407 | KF443382 | KF443390 | KF443398 | KF443394 |
| <i>R. japanensis</i> | MAFF 239636 T | KJ474829 | AB524621 | – | AB539114 | AB539101 |
| <i>R. kunmingensis</i> | HKAS 101773 | MH453491 | MH453487 | – | MH453480 | MH453484 |
| <i>R. margidorensis</i> | MUT 5329 T | KU314944 | MN556322 | MN556309 | MN605897 | MN605917 |
| <i>R. mediterranea</i> | MUT 5306 | KU255054 | MN556323 | MN556310 | MN605898 | MN605918 |
| <i>R. mexicana</i> | CPC25355 T | KT950848 | KT950862 | – | – | – |
| <i>R. neopustulans</i> | MFLUCC 11-0609 T | KJ474833 | KJ474841 | – | KJ474850 | – |
| <i>R. neopustulans</i> | MFLUCC 12-0003 T | KU940130 | KU863119 | KU872122 | – | – |
| <i>R. nitidula</i> | MFLUCC 11-0182 T | KJ474835 | KJ474843 | – | KJ474852 | KJ474859 |
| <i>R. nitidula</i> | MFLUCC 11-0634 T | KJ474834 | KJ474842 | – | KJ474851 | KJ474858 |
| <i>R. padinae</i> | MUT 5341 | KU158153 | MN556325 | – | MN605900 | MN605920 |
| <i>R. padinae</i> | MUT 5365 | KU158170 | MN556326 | KU158179 | MN605901 | MN605921 |
| <i>R. padinae</i> | MUT 5503 | KU314993 | MN556327 | MN556312 | MN605902 | MN605922 |
| <i>R. pseudohysterioides</i> | MFLUCC 13-0852 T | KU940131 | KU863120 | – | KU940198 | – |
| <i>R. pustulans</i> | KT 1709 | – | AB524623 | AB524482 | AB539116 | AB539103 |

| Species | Strain numbers | GenBank accession numbers | | | | |
|---------------------------------|------------------|---------------------------|----------|----------|----------------|-------------|
| | | ITS | LSU | SSU | <i>tef1- α</i> | <i>rpb2</i> |
| <i>R. scabrispora</i> | MFLUCC 11-0624 | KJ474836 | KJ474844 | – | KJ474853 | KJ474860 |
| <i>R. siamensis</i> | GMB0317 | ON4617749 | ON461896 | – | ON505014 | ON505010 |
| <i>R. siamensis</i> | GMB0391 | ON505054 | ON505053 | – | ON505018 | ON505013 |
| <i>R. tosaensis</i> | KT 1659 | – | AB524625 | AB524484 | AB539117 | AB539104 |
| <i>R. tuberculata</i> | MFLUCC 13-0854 T | KU940132 | KU863121 | – | – | – |
| <i>R. verrucispora</i> | CBS 125434 T | KJ474832 | – | – | – | – |
| <i>R. yunnanensis</i> | HKAS 101762 T | MH453492 | MH453488 | – | MH453481 | – |
| <i>R. yunnanensis</i> | MFLUCC 12-0005 T | KJ739604 | KJ474847 | KJ739608 | KJ474855 | KJ474862 |
| <i>Xenorousoella triseptata</i> | MFLUCC 17-1438 | MT214343 | MT214437 | MT214391 | MT235767 | MT235804 |
| <i>X. triseptata</i> | KNUF-20-NI009 | LC719282 | LC719283 | LC723530 | LC723531 | LC723532 |

* Remarks: The letter T denotes ex-type isolates. The newly generated sequences, new species and synonymized isolates are indicated in bold font.

Sequence alignment and phylogenetic analyses

Based on the sequence data of recent publications (De Silva et al. 2020; Li et al. 2023) and BLAST search results, reference taxa were selected, and phylogenetic analyses were conducted using the combined LSU, SSU, ITS, *tef1-α* and *rpb2* sequence data. Sequence alignments were made with the MAFFT v. 7 online tool (<http://mafft.cbrc.jp/alignment/server>; 2016). Alignments were improved where necessary, and composite sequence alignments were obtained using MEGA v. 6.0.

RAXML and Bayesian analyses were carried out on the CIPRESS Science Gateway Portal (<http://www.phylo.org>) (Miller et al. 2010). Maximum likelihood analysis was performed by RAXML-HPC v.8 (Stamatakis 2014) with rapid bootstrap analysis, followed by 1000 bootstrap replicates and the GTRGAMMA substitution model. MrBayes was used to perform BI analysis on XSEDE 3.2.7 (Ronquist et al. 2012), with tree samples taken at every 1000th generation during the 5,000,000-generation run of four concurrent Markov chains. The first 25% of the trees were removed as part of the burn-in phase, and calculations for the Posterior Probability were made for the remaining 75% of the trees (PP) (Rannala and Yang 1996; Zhaxybayeva and Gogarten 2002). The phylogenetic tree was displayed using Fig Tree v1.4.0 (Rambaut 2012) and was modified in Microsoft Office PowerPoint v. 2013.

Preliminary screening for antibacterial activity

Preliminary screening for antibacterial activity was carried out following the methods of Htet et al. (2024). Antibacterial discs of ampicillin were used as a positive control for screening (Alam et al. 2019). Antibacterial activity against gram positive bacteria (*Bacillus subtilis*-TISTR 1248 and *Staphylococcus aureus*-TISTR Y4b), and gram-negative bacteria (*Escherichia coli* TISTR 527) were investigated using the agar plug diffusion method (Balouiri et al. 2016). Bacteria test organisms were obtained from Scientific and Technological Instrument Center, Mae Fah Luang University. Bacterial test organisms were sub cultured and grown on Nutrient Agar (NA) for 24 hours. After 24 hours of inoculation, 2–3 loops of the bacterial test organisms were transferred to the nutrient broth and incubated for 24 hours in a shaking incubator. Prior to adding microbial suspensions to the sterile Mueller-Hinton agar media, cell counts were performed on the suspensions (6.7×10^5 cells/mL), as detailed by Mapook et al. (2020). Fungal mycelium plugs from our isolates were transferred to a solid medium plate and allowed to grow at room temperature for 24–48 hours. Inhibition zones were measured and compared to the positive control.

Results

Phylogenetic analyses

The combined LSU, SSU, ITS *tef1-α*, and *rpb2* sequence dataset comprises 66 taxa with *Occultibambusa bambusae* strains (MFLUC 13-0855 and MFLUCC 11-0394) as the outgroup taxa. Maximum likelihood (ML) analyses and Bayesian Inference (BI) of the combined dataset were performed to determine the placement of our new isolates. Tree topologies of ML and BI criteria were similar to earlier investigations (De Silva et al. 2020; Li et al. 2023). The best-scoring RAxML tree with a final likelihood value of -28736.822321 is shown in Fig. 1. RAxML analysis yielded 1578 distinct alignment patterns,

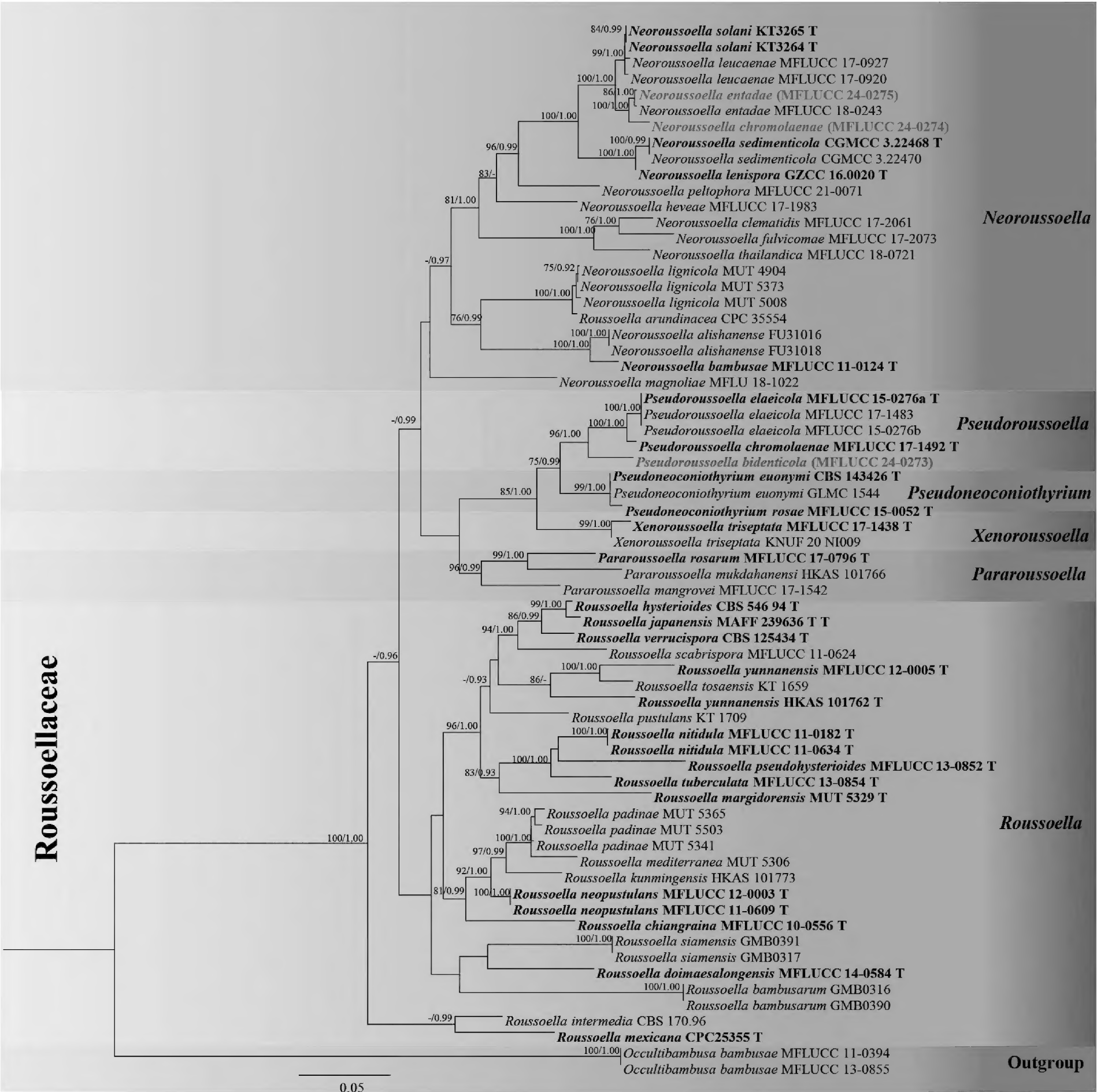


Figure 1. Phylogram generated from maximum likelihood analysis based on the combined dataset of LSU, SSU, ITS, *tef1-α* and *rpb2* sequence data. Bootstrap support values for ML equal to or greater than 75% and BYPP equal to or greater than 0.90 are given at the nodes. Newly generated sequences are in blue and type species are in bold.

with 29.39% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.245746, C = 0.258383, G = 0.266559, T = 0.229312; substitution rates: AC = 1.714222, AG = 4.957697, AT = 1.884927, CG = 1.345111, CT = 9.562411, GT = 1.000000; gamma distribution shape parameter $\alpha = 0.166563$. In our phylogenetic analysis, *Neorousoella chromolaenae* sp. nov. (MFLUCC 24-0274) clustered with *Neorousoella entadae* (MFLUCC 18-0243 and MFLUCC 24-0275) with 100% ML and 1.00 BYPP support. Furthermore, our isolate, *Pseudorousoella bidenticola* sp. nov. (MFLUCC 24-0273) formed a branch separated from *Ps. elaeicola* (MFLUCC 17-1483, MFLUCC 15-0276a, MFLUCC 15-0276b), and *Ps. chromolaenae* (MFLUCC 17-1492) with 96% ML and 1.00 BYPP, respectively.

Taxonomy

***Neorousoella chromolaenae* Z.H. Htet, A. Mapook & K. D. Hyde, sp. nov.**

Index Fungorum: IF902613

Facesoffungi Number: FoF16402

Fig. 2

Etymology. Name reflects the host plant *Chromolaena odorata*, from which this species was isolated.

Holotype. MFLU 24-0264.

Description. **Saprobic** on the dead stems of *Chromolaena odorata* (Asteraceae). **Sexual morph:** Undetermined. **Asexual morph:** Coelomycetous. **Conidiomata** 70–150 × 120–150 µm (av. 85 × 138 µm, n = 5), pycnidial, solitary, uniloculate, immersed, ostiolate. **Ostiole** papillate. **Peridium** 10–20 µm wide, comprising 2–3 layers of brown cells of **textura angularis**. **Conidiophores** reduced to conidiogenous cells. **Conidiogenous cells** 3–5 × 2–3.5 µm (av. 3 × 3 µm, n = 10), phialidic, ampulliform to cylindrical, hyaline. **Conidia** 3–6 × 2–4 µm (av. 4.4 × 3.1 µm, n = 20), hyaline, oblong to slightly ellipsoid, aseptate, with small guttules.

Culture characteristics. Conidia germinating on MEA within 24 hours, reaching 22 mm after 10 days at 27 °C, irregular, curled margin, brown in the middle and becoming pale brown on the outer parts of the culture, wrinkled on the surface; wrinkle, pale brown to brown in reverse.

Material examined. THAILAND • Chiang Rai Province, Doi Pui, 19°48'51"N, 99°52'1"E, on dead stems of *Chromolaena odorata* (Asteraceae), 14 March 2023, Zin Hnin Htet (CO-DP-3, MFLU 24-0264, holotype); ex-type culture MFLUCC 24-0274.

Notes. In a megablast search of GenBank, the closest match for the ITS sequence of our isolate was fungal sp. isolate NFC-3 (MG189955) with 99.47% similarity. The closest match for the LSU region was *N. solani* CBS 141288 (MH878207) with 100% similarity, and the closest match for the SSU region was *N. bambusae* strain GMB1295 (OM764650) with 93.99% similarity. Additionally, the closet matches for the *tef1-a* and *rpb2* gene regions were *Neorousoella entadae* strain MFLUCC 18-0243 (MK360065) and *N. entadae* strain MFLUCC 17-0920 (MK434898) with 99.45% and 99.77% similarities, respectively.

Based on the multi-locus phylogeny (Fig. 1), our isolate (MFLUCC 24-0274) formed a separate branch from *N. entadae* with 100% ML and 1.00 BYPP.

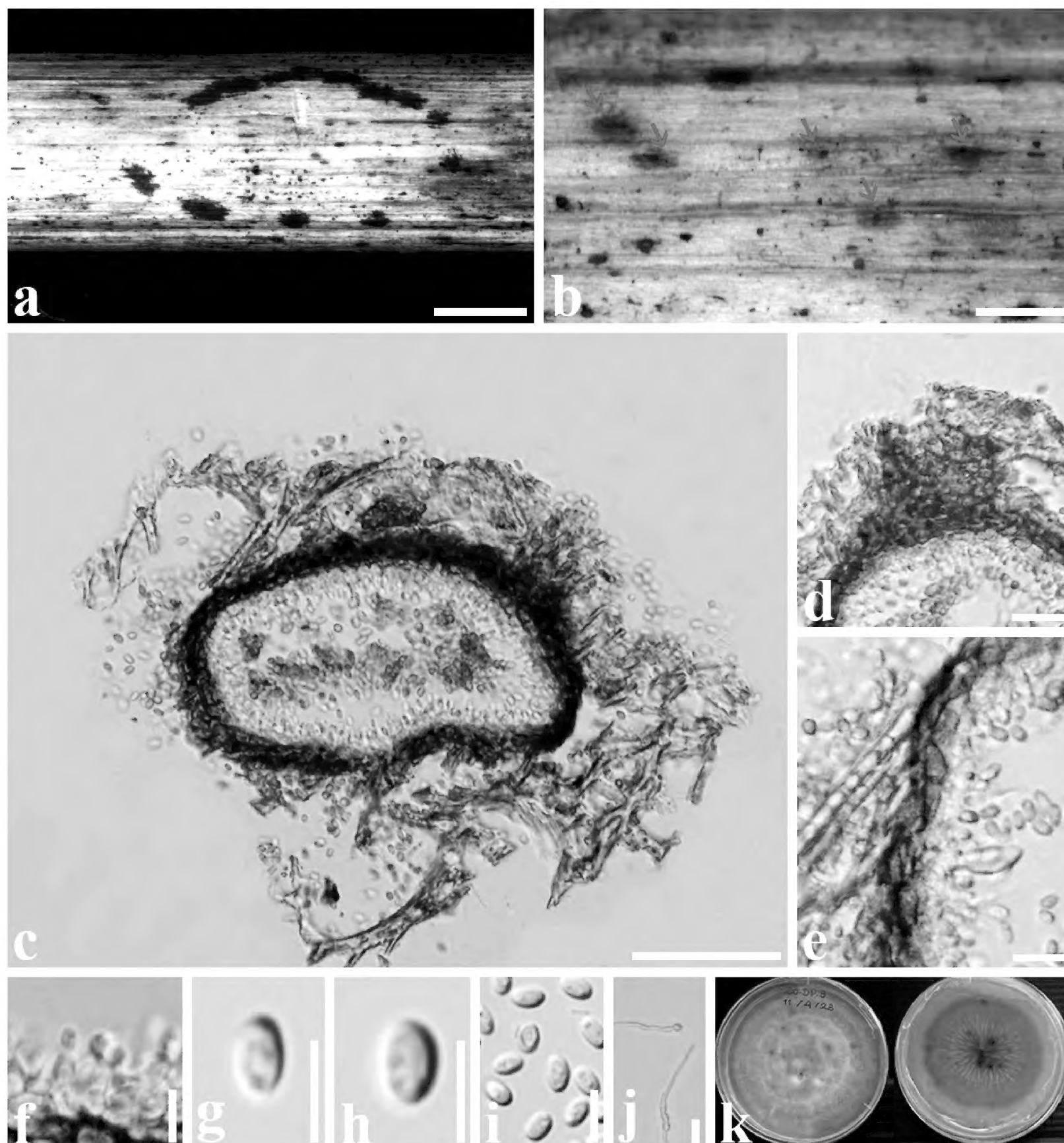


Figure 2. *Neorousoella chromolaenae* (MFLU 24-0264, holotype) **a, b** Conidiomata on the substrate **c** a section through conidioma **d** ostiole **e** peridium **f** conidia and conidiogenous cells **g–i** conidia **j** germinating conidia **k** culture on the MEA. Scale bars: 500 μm (**a, b**); 100 μm (**c**); 20 μm (**d, e**); 10 μm (**e–j**).

A comparative analysis of base pair differences between *Neorousoella chromolaenae* (MFLUCC 24-0274) and *Neorousoella entadae* (MFLUCC 18-0243) revealed variations in ITS (0.6% - 3/476), LSU (0.1% - 1/838), SSU (1.9% - 14/717), *tef1- α* (0.5% - 5/902), and *rpb2* (2.0% - 18/885) without gaps, respectively. Morphologically, our collection is similar to *N. entadae* (MFLUCC 17-0920) in having solitary, unilocular, ostiolate, phialidic, ampulliform to cylindrical, hyaline conidiogenous cells, and oblong to ellipsoidal, hyaline conidia (Jayasiri et al. 2019). However, our species differs from *N. entadae* (MFLUCC 17-0920) in having smaller conidiomata (70–150 \times 120–150 μm vs. 127–192 \times 161–190 μm), slightly wider conidiogenous cells (2–3.5 μm vs. 0.7–1.8 μm) and larger conidia size (3–6 \times 2–4 μm vs. 3–4 \times 1.7–1.9 μm). Therefore, *N. chromolaenae* is described here as a new species based on phylogeny and morphological evidence. Synopsis of the asexual morph of *Neorousoella* species is also provided in Table 3.

Table 3. Synopsis of asexual morph species in *Neorousoella*.

| Species | Conidiomata (µm) | Conidiogenous cells (µm) | Conidia (µm) | References |
|---|-------------------|--------------------------|-------------------|----------------------------|
| <i>Neorousoella alishanense</i> (FU31016) | 130–140, 210–225 | 8–14 × 2–3 | 3–4 × 2–3 | Karunarathna et al. (2019) |
| <i>Neorousoella bambusae</i> (MFLUCC 11-0124) | 200–430 × 300–420 | 8–13.5 × 1.5–3 | 3–4 × 1.5–2 | Liu et al. (2014) |
| <i>Neorousoella chromolaenae</i> (MFLUCC 24-0274) | 70–150 × 120–150 | 3–5 × 2–3.5 | 3–6 × 2–4 | This study |
| <i>Neorousoella entadae</i> (MFLUCC 17–0920) | 127–192 × 161–190 | 3.5–5.6 × 0.7–1.8 | 3–4 × 1.7–1.9 | Jayasiri et al. (2019) |
| <i>Neorousoella entadae</i> (MFLUCC 24-0275) | 70–120 × 100–150 | 3–5 × 1–3 | 3–5 × 2–4 | This study |
| <i>Neorousoella heveae</i> (MFLUCC 17-0338) | 90–130, 115–180 | 3–7 × 2–5 | 2.5–5 × 2–4 | Phookamsak et al. (2019) |
| <i>Neorousoella leucaenae</i> (MFLUCC 18–1544) | 135–175 × 120–180 | 5.5–9 × 3–4 | 3.5–4.5 × 1.9–2.6 | Jayasiri et al. (2019) |
| <i>Neorousoella peltophora</i> (MFLUCC 21-0071) | 165–224 × 144–178 | 1–3.5 × 6.5–8 | 2.0–3.0 × 3.0–4.0 | Pasouvang et al. (2022) |
| <i>Neorousoella solani</i> (CPC 26331) | To 150 | 4–6 × 3–4 | 4.5–5 × 2–3 | Crous et al. (2016) |

***Neorousoella entadae* Jayasiri, E.B.G. Jones & K.D. Hyde, Mycosphere 10(1): 105 (2019)**

Index Fungorum: IF555568

Facesoffungi Number: FoF05275

Fig. 3

Description. *Saprobic* on the dead stems of *Chromolaena odorata* (Asteraceae). **Sexual morph:** Undetermined. **Asexual morph:** Coelomycetous. **Conidiomata** 70–120 × 100–150 µm (av. 95 × 110 µm, n = 5), pycnidial, solitary, globose to subglobose, uniloculate, immersed to semi-immersed, ostiolate. **Ostiole** 30–35 µm wide, with small papillate. **Peridium** 10–20 µm wide, comprising 2–3 layers of brown cells of *textura angularis*. **Conidiophores** reduced to conidiogenous cells. **Conidiogenous cells** 3–5 × 1–3 µm (av. 3.8 × 2.1 µm, n = 10), phialidic, ampulliform to cylindrical, hyaline. **Conidia** 3–5 × 2–4 µm (av. 3.8 × 2 µm, n = 20), hyaline, becoming pale brown when gathering, oblong to ovoid, aseptate, with small guttules.

Culture characteristics. Conidia germinating on PDA within 24 hours, reaching 24 mm after 7 days at 27 °C, irregular, entire, yellowish-brown, slightly wrinkled on the surface; pale brown to brown in reverse.

Material examined. THAILAND • Chiang Rai Province, Thoeng district, on dead stems of *Chromolaena odorata* (Asteraceae), 24 Jan 2022, A. Mapook (TCR18, MFLU 24-0265, new host record); living culture MFLUCC 24-0275.

Known host distribution. *Entada phaseoloides* (Fabaceae), *Leucaena* sp. (Fabaceae) (Jayasiri et al. 2019).

Notes. In a BLASTn search of GenBank, the closest match for the ITS sequence of our isolate was *N. solani* strain MnF107 (OQ704272) with 99.83% similarity. The closest match for the LSU region was *Rousoella* sp. strain HF3S53 (OP179275) with 99.77% similarity, and the closest match for the SSU region was *Pleosporales* sp. IRB20-2 (AB195632) with 100% similarity. The closest match for the *tef1-a* and *rpb2* gene region was *Neorousoella entadae* strain MFLUCC 18-0243 (MK434866) with 99.78% and 99.53% similarity, respectively.

When we compared the morphology, our isolate is similar to *N. entadae* (MFLUCC 17–0920) in having solitary, unilocular, ostiolate conidiomata, phialidic, ampulliform to cylindrical, hyaline conidiogenous cells, and oblong to ovoid, hyaline conidia with size (3–5 × 2–4 µm vs 3–4 × 1.7–1.9 µm). However, our isolate differs from *N. entadae* (MFLUCC 17–0920) in having smaller

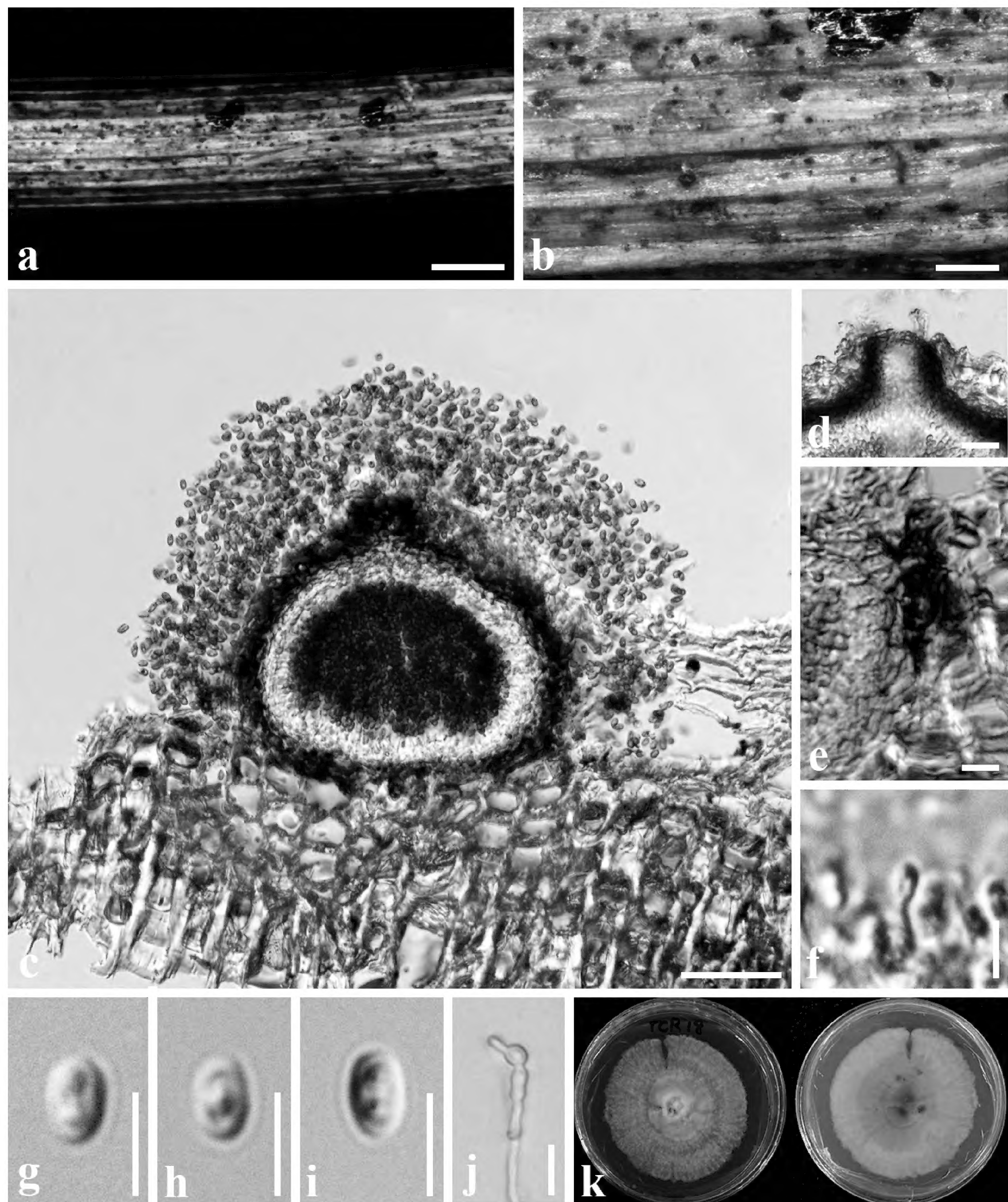


Figure 3. *Neorousoella entadae* (MFLU 24-0265, new host record) **a, b** Conidiomata on the substrate **c** a section through conidioma **d** ostiole **e** peridium **f** conidia and conidiogenous cells **g–i** conidia **j** a germinating conidium **k** culture on the MEA. Scale bars: 500 μm (**a**); 200 μm (**b**); 50 μm (**c**); 20 μm (**d, e**); 5 μm (**f, g, h, i, j**).

conidiomata (70–120 \times 100–150 μm vs. 127–192 \times 161–190 μm), slightly wider conidiogenous cells (3–5 \times 1–3 μm vs. 3.5–5.6 \times 0.7–1.8 μm) (Table 3).

Based on the multi-locus phylogeny (Fig. 1), our isolate MFLUCC 24-0275 clustered in the same clade with *N. entadae* (MFLUCC 18-0243). Moreover, there is no significant base pair difference between MFLUCC 24-0275 and *N. entadae* (MFLUCC 18-0243). Therefore, we reported *N. entadae* as the first occurrence on *C. odorata* (Asteraceae) based on morphology and multigene phylogeny.

***Pseudorousoella bidenticola* Z.H. Htet, A. Mapook & K. D. Hyde, sp. nov.**

Index Fungorum: IF902614

Facesoffungi Number: FoF16403

Fig. 4

Etymology. Name reflects the host plant *Bidens pilosa*, from which this species was isolated.

Holotype. MFLU 24-0266.

Description. *Saprobic* on dead stems of *Bidens pilosa*. **Sexual morph:** Undetermined. **Asexual morph:** Coelomycetous. **Conidiomata** 120–150 × 150–180 µm (av. 126 × 173 µm, n = 5), pycnidial, solitary, immersed to semi-immersed, uni-loculate, brown, globose to subglobose, dark fruiting bodies on the host substrate, without an ostiole. **Peridium** 10–20 µm wide, comprising 2–3 layers of yellowish brown to brown cells of *textura angularis*. **Conidiophores** reduced to conidiogenous cells. **Conidiogenous cells** 1–2 µm long, holoblastic, short, globose to subglobose, hyaline and unbranched. **Conidia** 5–7.5 × 4–5.5 µm (av. 6 × 4.8 µm, n = 20), globose to subglobose, brown to reddish brown, aseptate, thick-walled with a guttule.

Culture characteristics. Conidia germinating on MEA within 24 hours, reaching 27 mm after 10 days at 27 °C, irregular, entire, concentric, opaque, flat, white to pale brown on the surface; concentric, creamy to pale brown in reverse.

Material examined. THAILAND • Chiang Rai Province, Doi Pui, 19°48'51"N, 99°52'1"E, on dead stems of *Bidens pilosa* (Asteraceae), 14 March 2023, Zin Hnin Htet (BP-DP-11, MFLU 24-0266, holotype); ex-type culture MFLUCC 24-0273.

Notes. In a BLASTn search of GenBank, the closest match for the ITS sequence of our isolate was *Rousoella elaeicola* strain MFLUCC 15-0276b (MH742330) with 94.57% similarity. The closest match for the LSU region was *Pseudorousoella chromolaenae* isolate MFLUCC 17-2062 (MT394704) with 92.95% similarity, and the closest match for the SSU region was *Parathyridaria tyrrhenica* MUT<ITA>:5371 (KU314952) with 99.16% similarity. Additionally, the closest matches for the *tef1-α* and *rpb2* gene regions were *Pseudorousoella elaeicola* culture MFLUCC:17-1483 (MT235772) and *Rousoella* sp. strain GMB1153 (OM755588) with 97.08% and 98.27% similarity, respectively.

Pseudorousoella elaeicola (MFLUCC 17-1483 and MFLUCC 17–2086) was found as a sexual morph in nature (Phookamsak et al. 2019, Mapook et al. 2020); hence, we were unable to directly compare their morphology with our isolate. However, based on comparing the morphology of *Pseudorousoella bidenticola* (MFLUCC 24-0273) and *Ps. chromolaenae* (MFLUCC 17-1492), our species differs from *Ps. chromolaenae* (MFLUCC 17-1492) in having immersed to semi-immersed, globose to subglobose, brown, conidiomata without ostiole, smaller-sized (120–150 × 150–180 µm vs 130–175(–230) × 160–230 µm), holoblastic, globose to subglobose conidiogenous cells, and brown to reddish brown, globose to subglobose conidia with guttules, while *Ps. chromolaenae* (MFLUCC 17-1492) displays superficial, globose to obpyriform, yellowish brown to brown conidiomata with a central ostiole, annellidic, ampulliform to oblong conidiogenous cells, and oblong to oval, conidia that are pale brown to light brown when immature, becoming yellowish brown to reddish brown when mature (Table 4).

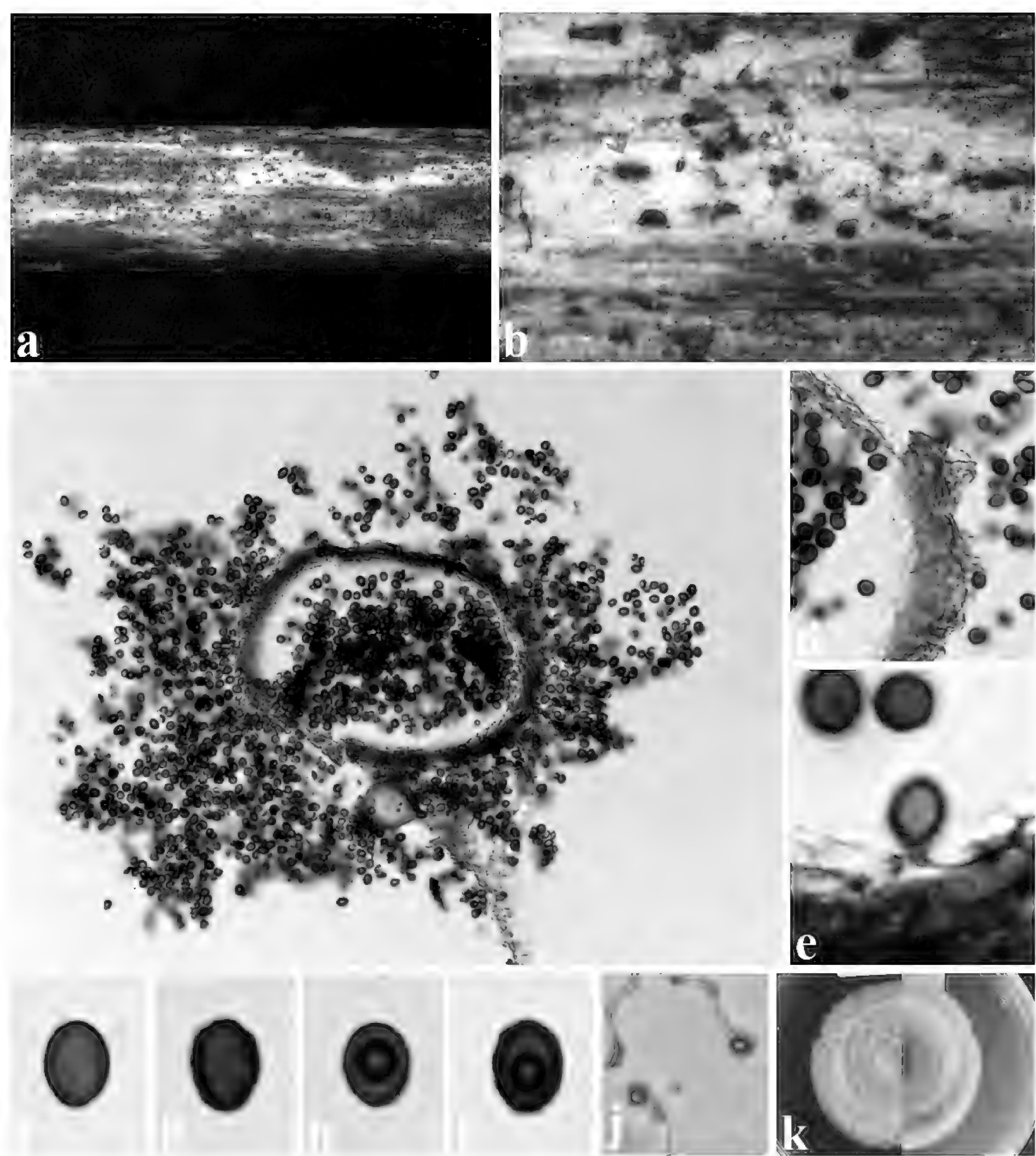


Figure 4. *Pseudorousoella bidenticola* (MFLU 24-0266, holotype) **a, b** Conidiomata on the substrate **c** a section through a conidioma **d** peridium **e** conidia and conidiogenous cell **f–i** conidia **j** germinating conidia **k** culture on the MEA. Scale bars: 500 µm (**a, b**); 100 µm (**c**); 10 µm (**d**); 5 µm (**e–i**).

Table 4. Synopsis of sexual and asexual morph of *Chromolaenicola* species.

| Species | Conidiomata (µm) | Conidiogenous cells (µm) | Conidia (µm) | References |
|--|-------------------------|--------------------------|-----------------|----------------------|
| <i>Pseudorousoella chromolaenae</i> (MFLUCC 17-1492) | 130–175(–230) × 160–230 | – | 5.5–7 × 3.5–5 | Mapook et al. (2020) |
| <i>Pseudorousoella bidenticola</i> (MFLUCC 24-0273) | 120–150 × 150–180 | 1–2 | 5–7.5 × 4–5.5 | This study |
| Species | Ascomata (µm) | Asci (µm) | Ascospores (µm) | References |
| <i>Pseudorousoella elaeicola</i> | 225–475 × 240–400 | 95–135 × 6–8.5 | 10–14 × 4.5–6 | Mapook et al. (2020) |

Based on the multi-locus phylogeny (Fig. 1), our isolate (MFLUCC 24-0273) formed a separate branch related to *Pseudorousoella* species with 96% ML and 1.00 BYPP. When comparing base pair differences between *Ps. bidenticola* (MFLUCC 24-0273) and *Ps. chromolaenae* (MFLUCC 17-1492), variations

Table 5. Preliminary antibacterial activity result of this study.

| Species | Zone of inhibition (mm); Ampicillin (+) | | |
|---|---|-------------------------|------------------------------|
| | <i>Bacillus subtilis</i> | <i>Escherichia coli</i> | <i>Staphylococcus aureus</i> |
| <i>Neorousoella chromolaenae</i> (MFLUCC 24-0274) | 16 mm inhibition | 11 mm inhibition | 20 mm inhibition |
| <i>N. entadae</i> (MFLUCC 24-0275) | 13 mm inhibition | 17 mm inhibition | 14 mm inhibition |
| <i>Pseudorousoella bidenticola</i> (MFLUCC 24-0273) | 18 mm inhibition | 12 mm inhibition | 13 mm inhibition |

Abbreviation: Positive control (+).

were observed in ITS (3.6% - 23/469), LSU (0.6% - 5/799), SSU (0.6% - 4/630), *tef1-α* (2.6% - 24/891), without gaps. Therefore, we introduced our collection (MFLUCC 24-0273) as a new species based on morphology and multigene phylogeny. Moreover, this is also the first record of *Pseudorousoella* species from *Bidens pilosa* (Asteraceae).

Preliminary screening for antibacterial activity

In our study, we explored the antibacterial activities of our three isolates against *Bacillus subtilis*, *Escherichia coli coli*, and *Staphylococcus aureus*. *Neorousoella chromolaenae* (MFLUCC 24-0274), *N. entadae* (MFLUCC 24-0275), *Pseudorousoella bidenticola* (MFLUCC 24-0273) exhibited antibacterial activity against all three test organisms. For *B. subtilis*, *N. chromolaenae* (MFLUCC 24-0274), *N. entadae* (MFLUCC 24-0275), and *Ps. bidenticola* (MFLUCC 24-0273) exhibited partial inhibition. Against *E. coli*, *N. chromolaenae* (MFLUCC 24-0274), *N. entadae* (MFLUCC 24-0275), and *Ps. bidenticola* (MFLUCC 24-0273) demonstrated clear inhibition. For *S. aureus*, *N. chromolaenae* (MFLUCC 24-0274) showed the most significant inhibition, followed by *N. entadae* (MFLUCC 24-0275) and *Ps. bidenticola* (MFLUCC 24-0273), with clear inhibition observed. However, none of these fungal species showed a wider inhibition zone than the positive control, ampicillin (20 mm for *B. subtilis*, 50 mm for *E. coli*, 40 mm for *S. aureus*). The measurements of clear inhibition zones produced by our new isolates are provided in Table 5.

Discussion

Our research in northern Thailand unveiled the introduction of two novel species and one new host record within the Rousoellaceae. This classification was determined through a combination of morphological analyses and a multigene phylogeny approach, adhering to the recommendations outlined by Jeewon and Hyde(2016). Mapook et al. (2020) established *Pseudorousoella* to accommodate *Ps. chromolaenae* and *Ps. elaeicola*. Interestingly, our study revealed a third *Pseudorousoella* strain on the dead stems of *Bidens pilosa* (Asteraceae). In our phylogenetic analyses, our isolate (MFLUCC 24-0273) formed a basal lineage to other strains (MFLUCC 17-1483; MFLUCC 15-0276a; MFLUCC 15-0276b and MFLUCC 17-1492). Our species is morphologically similar to *Ps. chromolaenae* and has a significant base pairs difference between the two, and herein, we introduced our isolate as a new species. Moreover, we found two isolates of *Neorousoella* on the dead stems of *C. odorata* (Asteraceae). Based on the morphological similarity and multigene phylogeny, we identified *N. chromolaenae* as a new species and *N. entadae* as the first occurrence on *Chromolaena odorata*.

Following the preliminary screening for antibacterial activity, all species examined in our study demonstrated potential antibacterial properties. In a prior examination of *Pseudorousoella* species, *Ps. chromolaena* exhibited no inhibition on *B. subtilis*, *E. coli*, and *M. plumbeus*, while *Ps. elaeicola* displayed a 10 mm inhibition zone against *E. coli* (Mapook et al. 2020). Our isolate (MFLUCC 24-0273) exhibited inhibition on all tested organisms, with inhibition zones measuring 18 mm (*B. subtilis*), 12 mm (*E. coli*), and 13 mm (*S. aureus*), respectively. These results highlight our new species, *Pseudorousoella bidenticola*, as a particularly promising organism for further research. Additionally, *Neorousoella chromolaenae* (MFLUCC 24-0274) and *N. entadae* (MFLUCC 24-0275) demonstrated inhibitory effects against all test organisms, and specific measurements of inhibition zones are shown in Table 4. Across the three observed species, all species exhibited antibacterial activity. The outcomes of our investigation suggest that this fungal group possesses antibacterial potential, presenting a source for the exploration of novel bioactive compounds. These findings also highlight the potential of the Roussoellaceae family for antibacterial compound discovery, a field that remains relatively underexplored.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

The authors confirm contributions to the paper as follows: Fungal specimen collection and isolation, fungal specimen deposition and manuscript writing: Zin Hnin Htet; fungal identification and contributed in the revision of the manuscript: Zin Hnin Htet, Kevin D. Hyde, Fatimah O. Alotibi, Ausana Mapook, Thilini K. W. Chethana. All authors have read and agreed to the published version of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text.

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